Treatment of Murine Hemangioendotheliomas With the Angiogenesis Inhibitor AGM-1470

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● **Hemangioma and other angiomatous diseases of childhood are common. Although most lesions are harmless and self-limiting, some are associated with significant morbidity and may be life-threatening. Interferon-α, a weak angiogenesis inhibitor, recently has been found to significantly reduce the mortality rate associated with life-threatening hemangiomas. The effectiveness of AGM-1470, a potent inhibitor of angiogenesis derived from the fungal product fumagillin, was tested in a mouse model of hemangioendothelioma. Thirty syngeneic mice were implanted with cells derived from a spontaneous mouse hemangioendothelioma. Tumors formed within 2 to 3 days, and the animals were then treated systemically with AGM-1470 or with saline and vehicle alone. After 22 days, the tumor volume in the saline-treated mice was 7368 ± 2723 mm^3, versus 709 ± 73 mm^3 in the mice that received AGM-1470 ($P < .001$). Survival was prolonged for the AGM-1470-treated mice, and there was no evidence of drug-related toxicity. All experiments were repeated. In this study, AGM-1470 was safe and highly effective in the treatment of hemangioendothelioma. AGM-1470, and other antiangiogenic agents, may provide safe and effective treatment for hemangioma and other angiomatous diseases.**

INDEX WORDS: Angiogenesis; AGM-1470; TNP-470; antiangiogenic therapy; hemangioma; hemangioendothelioma; angiomatous disease.

ANGIOMATOUS diseases, which include hemangiomas, hemangioendotheliomas, and related disorders, can affect up to 3% of all children.[1-3] These lesions can lead to complications, including hemorrhage, thrombocytopenia, and congestive heart failure, and can be fatal. Angiomatous lesions also can destroy or disfigure tissues by displacing the normal architecture. If this occurs in the eye or in periocular tissues, vision can be impaired or lost. The purpose of this study was to determine whether AGM-1470 would be useful in the treatment of a mouse tumor model of hemangioendothelioma, called EOMA.[4-8] EOMA is a spontaneously occurring mouse hemangioendothelioma that can arise in the subcutaneous middorsum of 129/J mice. EOMA cells retain many of the characteristics of microvascular endothelial cells. When implanted into syngeneic mice, EOMA cells readily form large hemangioendotheliomas. These tumors are highly angiogenic and grow rapidly. If left untreated, they will be fatal.

Angiogenesis,[9] the growth of new blood vessels, occurs normally and plays an important role in many physiological and pathological states.[10] Neovascularization is crucial for the growth of tumors beyond a few cubic millimeters.[11,12] Thus, one strategy for the treatment of carcinoma would be to use antiangiogenic agents to inhibit tumor neovascularization. AGM-1470 (Fig 1), also known as TNP-470, is one such agent. AGM-1470 is a synthetic analog of fumagillin, which was first isolated from the fungus *Aspergillus fumigatus* fresnius.[13,14] The fungus was first found in our laboratory as a contaminant of endothelial cell cultures. AGM-1470 has been shown to be a potent inhibitor of angiogenesis in both in vitro and in vivo studies.[15] In vitro, the proliferation and the migration of endothelial cells are reversibly inhibited by AGM-1470 at log doses lower than for other cell types. In animal models,[16-21] AGM-1470 is highly effective in the treatment of a wide variety of tumors and their metastases.[22] Further, AGM-1470 is highly effective in an animal model of collagen arthritis[23] and in the treatment of corneal[24] and iris[25] neovascularization in rabbits and monkeys, respectively. Recently,[26-28] AGM-1470 and other angiogenesis inhibitors, in combination with each other or with cytotoxic agents, have been shown to improve efficacy in the treatment of primary tumors and metastatic disease. In the present study, we used AGM-1470 to treat an angiomatous disease. This is the first report of the use of AGM-1470 in the treatment of an experimental model of angiomatous disease.

MATERIALS AND METHODS

Cell Culture

The EOMA cell line was provided by R. Auerbach (University of Wisconsin, Madison, WI) and C. Meinenger (Texas A&M University, College Station, TX). The line was maintained in culture in Dulbecco's modified eagle's medium, supplemented with 10% heat-inactivated (56°C × 20 minutes) bovine calf serum and antibiotics. Stock solutions of glutamine and antibiotics were obtained from Irvine Scientific (Santa Ana, CA). Media, serum, and

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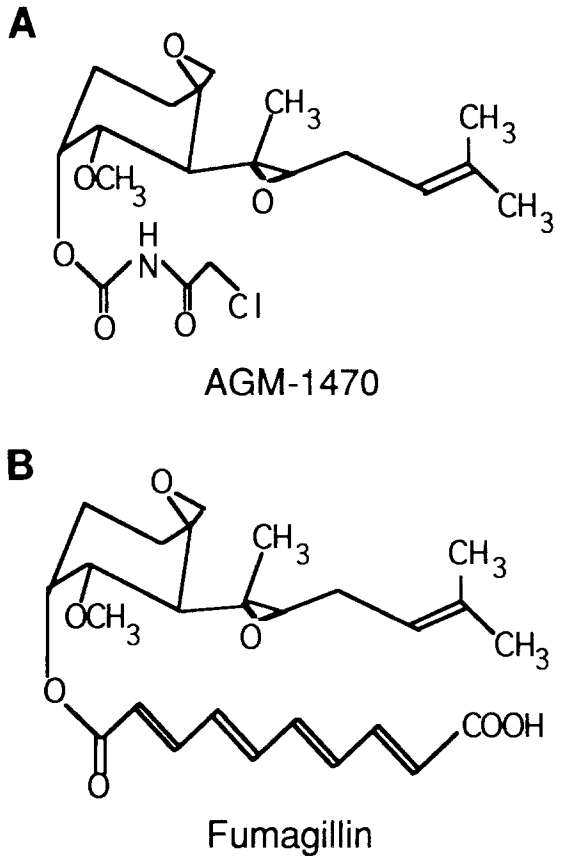

Fig 1. Structure of AGM-1470. (A) A potent inhibitor of angiogenesis that is a synthetic analogue of fumagillin. (B) The parent compound isolated from *Asperigillus fumigatus*.

phosphate-buffered saline were obtained from JRH Biosciences (Lenexa, KS).

Cell Preparation

On the day of tumor implantation, the EOMA cells in culture were washed with phosphate-buffered saline and were trypsinized with 0.05% trypsin/EDTA (Life Technologies, Inc, Grand Island, NY). The cells were resuspended in media and centrifuged for 10 minutes at 1,000 rpm. The cell pellet was resuspended with chilled saline to achieve a final concentration of 5×10^6 cells/mL. The cell suspension was kept in an ice bath at all times.

Animal Studies

Three- to five-week-old 129/J male mice (Jackson Laboratories, Bar Harbor, ME) were injected subcutaneously with 5×10^5 tumor cells. The mice were acclimated for several days before experimentation, and their backs were shaved to facilitate tumor implantation and measurement. All mice were monitored daily, and the tumors, when first visible and palpable (80 to 160 mm^3), were measured in two dimensions (length and width) using calipers. Tumor volume was calculated[29] using the following formula: (length) $\times$ (width)$^2 \times$ (Π/6), and the mice were randomized into two groups. All mice were individually caged. One group received AGM-1470 at a dose of 30 mg/kg starting when tumors were first noted. Subcutaneous injections were made at a site distant from the tumor every second day for the course of the experiment. The other group received injections of a comparable volume of vehicle alone. All injections were given via a 30-gauge needle. Every second day, the tumor dimensions were measured and the mice were weighed. Tumor volume was calculated as described above, and the ratio of tumor size in the treated animals to tumor size in the control animals was calculated (T:C ratio). Statistical analysis was performed using the Student's *t* test.

Anesthesia

The animals were anesthetized in a methoxyfluorane chamber before shaving, tumor implantation, and photography. After anesthesia, the animals were observed until they had fully recovered. They were killed by continuous inhalation of methoxyfluorane.

AGM-1470

AGM-1470 was provided by Takeda Chemical Industries, Ltd (Osaka, Japan) and stored dry at −20°C. A stock solution of 10% (wt/vol) AGM-1470 in ethanol was made monthly and kept at 4°C. Immediately before injections, a treatment solution was made by diluting the stock solution with 0.9% normal saline (20 μL stock solution per milliliter of saline, in a polypropylene tube). A control solution of vehicle alone was made by adding 2% (vol/vol) ethanol to 0.9% normal saline.

RESULTS

Tumor Growth

Two days after implantation, the tumor volume in the control group was 134 ± 10 mm^3, versus 134 ± 12 mm^3 in the treatment group. After 22 days of treatment (n = 15 mice per group), the tumor volume in the group that received saline and vehicle alone was 7368 ± 2723 mm^3, versus 709 ± 73 mm^3 in the group treated with AGM-1470 ($P < .001$) (Fig 2). The T:C ratio decreased progressively during the experiment, and was 0.1 on day 22 of treatment (Fig 3), representing a 10-fold smaller primary tumor in the AGM-1470 treated mice (Fig 4). The experiment was repeated, and the results were comparable (data not shown).

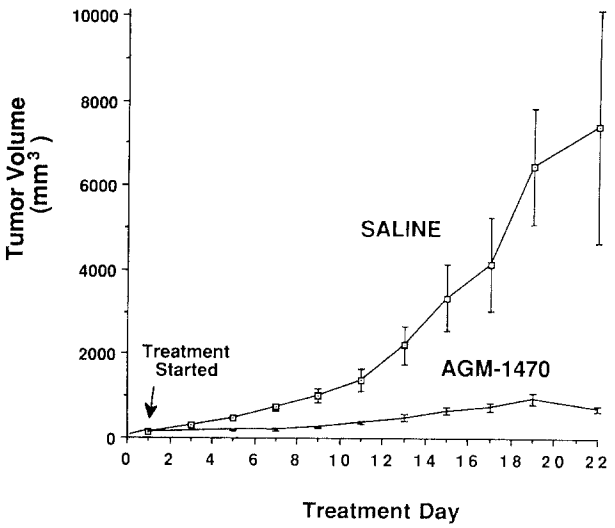

Fig 2. Inhibition of the growth of mouse hemangioendotheliomas, EOMA, by systemic treatment with AGM-1470.

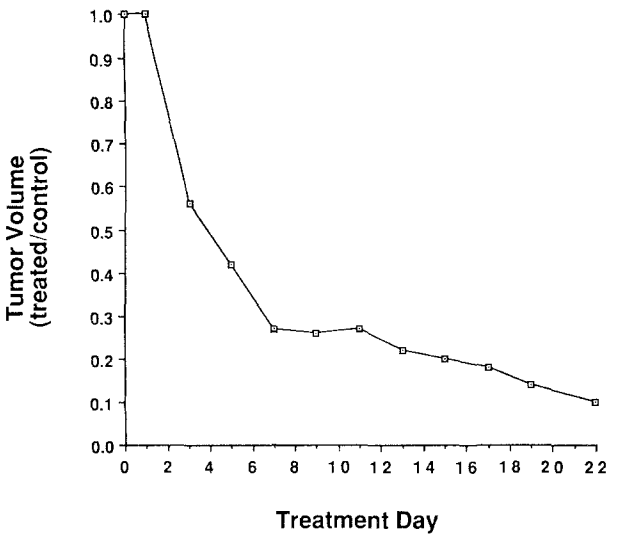

Fig 3. Decreasing ratio of hemangioendothelioma size among animals treated with AGM-1470 after implant versus saline and vehicle alone (T:C ratio).

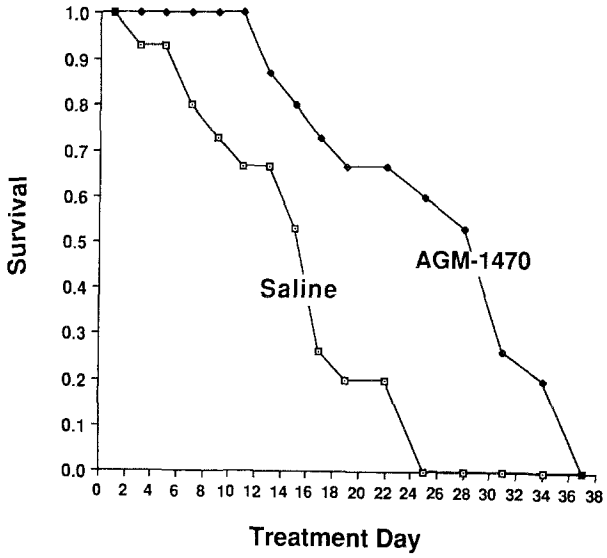

Fig 5. Prolongation of survival of mice implanted with hemangioendotheliomas, by systemic treatment with AGM-1470.

Survival

Twenty-two days after treatment began, the survival rate was 20% for the control group and 67% for the group treated with AGM-1470 (Fig 5). The mice treated with AGM-1470 survived 1.7 times longer than those in the control group, but all eventually died because of hemorrhagic complications, which were present in both groups. The mice were bleeding locally from their tumors shortly before dying. Autopsy of all mice showed hemorrhage and thrombosis of the primary tumors, but no evidence of metastasis or other pathology. All mice gained weight throughout the study and had no evidence of drug-related toxicity (eg, diminished activity, hair loss, anorexia, diarrhea or neurological abnormalities).

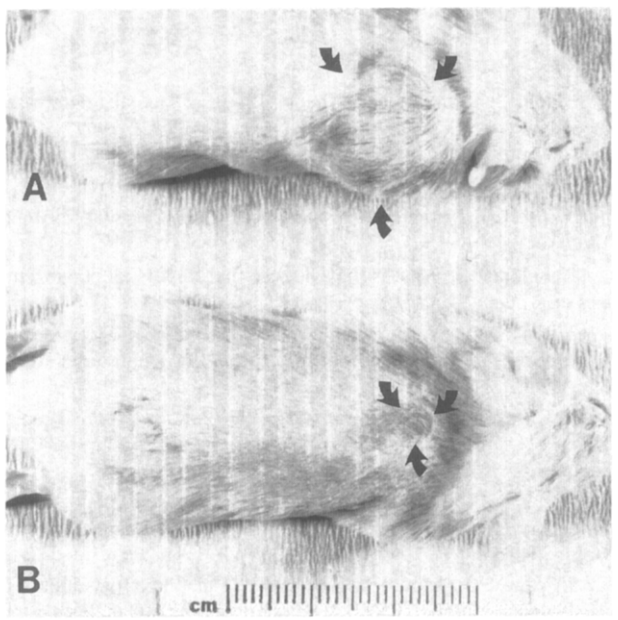

Fig 4. Reduction of hemangioendothelioma size by systemic treatments with saline and vehicle alone (A) or AGM-1470 (B) after tumor implantation. Arrows denote the primary tumor.

DISCUSSION

Through this study, we have shown that AGM-1470, a potent inhibitor of angiogenesis, is highly effective in the treatment of an angiomatous disease. Pediatric angiomatous lesions of childhood are common and only rarely cause significant problems. For hemangioma, which is the most common tumor of infancy, the incidence is 1% to 2% among neonates, and increases to 12% by the age of 1 year.[30,31] In some cases, perhaps 10% in cases of hemangioma, these lesions can lead to severe complications and even death. Previously,[32] therapy for these lesions has included pharmacological treatment with high-dose corticosteroids[33] or chemotherapeutic agents such as cyclophosphamide.[34] Other therapies include embolization,[35] radiation therapy,[36] laser therapy,[37] surgical resection, and combined modalities. Such treatments often lead to significant morbidity and have a relatively high failure rate. For these reasons, safe and highly effective treatments for life-threatening angiomatous disease are needed. In cases of hemangioma, interferon-α, which may be working by inhibiting angiogenesis of the lesions,[38] has been used recently.[39-43] It has been highly effective when other therapies have failed, with only little toxicity. However, it is not a potent angiogenesis inhibitor and requires prolonged therapy. Thus, there is a need for

improvement in the treatment of serious childhood hemangioma and of other angiomatous diseases.

We have shown that the angiogenesis inhibitor AGM-1470 is highly effective in the treatment of a mouse model of angiomatous disease. AGM-1470 dramatically and reproducibly inhibited the growth of hemangioendotheliomas and prolonged survival. We found that mice treated with AGM-1470 had 90% reduction in tumor size (T:C ratio = 0.1) and a 170% longer survival period. Prolonged therapy with AGM-1470 resulted in virtually no toxicity and continued inhibition of tumor growth. Despite this, all mice eventually died of hemorrhage directly from the primary tumor. These complications were present in both groups and occurred later in the AGM-1470 group. These findings of high efficacy of AGM-1470 and low toxicity are in agreement with reports of its use in other animal tumor systems and in a primate model of iris neovascularization. The low toxicity associated with AGM-1470 is further supported by in vitro findings showing that AGM-1470 inhibits proliferating endothelium but has little effect on quiescent endothelial cells.

The EOMA cell line and tumors we used are derived from a hemangioendothelioma that arose spontaneously and still maintains several characteristics of microvascular endothelium. Thus, the tumors are ideal for the study of angiomatous disease. Other animal models of angiomatous disease involve use of the middle-T oncogene.[44-47] In one model, transgenic mice expressing the polyoma middle-T antigen oncogene DNA develop transplantable angiomatous lesions. Alternatively, normal endothelial cells can be transfected in vitro with the middle-T oncogene. We implanted mice with several of these cell lines, including transgenic cells derived from brain endothelium (bENDO), and found that the cells had low tumorigenicity and early spontaneous regression. Other models of angiomatous disease include the F-2 cell line[48] that arose on the skin of a nude mouse exposed to ultraviolet light and the D14 hemangioendothelioma line[49] that arose in the liver of a nude mouse exposed to 1,2-dimethylhydrazine dihydrochloride. Because the EOMA cell line arose spontaneously in an immunocompetent mouse, is easily maintained, and reproducibly forms tumors when injected into syngeneic mice, it may correlate better with human angiomatous diseases than do other models.

Our results suggest that AGM-1470 may be very useful in the treatment of angiomatous diseases in humans. In animal models, AGM-1470 is more effective than interferon in the treatment of carcinoma and is a more potent inhibitor of angiogenesis. The use of AGM-1470, alone or with other modalities, should improve outcome and survival in cases of life-threatening hemangioma. Further, AGM-1470, or other nontoxic angiogenesis inhibitors, may be useful in the treatment of other vascular lesions of childhood. The use of antiangiogenic agents, such as AGM-1470, in the treatment of neoplasms and other pathological states is associated with improved outcome and decreased toxicity when compared with other modalities. In the future, potent angiogenesis inhibitors, either alone or in combination, may be more effective than existing modalities in the treatment of angiomatous disease.

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Discussion

R. Azizkhan (Buffalo, NY): Current treatment of morbid hemangiomas remains unsatisfactory. Unfortunately, steroids and alpha-interferon are not always effective and are clearly associated with significant side effects. Additional strategies and approaches to the treatment of angiomatous diseases are needed. This study provides animal experimental evidence that may provide a very unique way of treating this type of problem.

I have a few questions for the authors. Mast cells

have been observed in rapidly proliferating hemangiomas and are absent from involuting or stable lesions. Have you looked at the presence of mast cells in this type of model? Is there any effect of AGM-1470 on mast cells? In your study, the AGM-1470 appears to ameliorate the course of disease but not prevent the eventual fatal outcome associated with hemangioma. Do you have any further comments about this observation? Does it make a difference in how you provide the agent, continuous versus pulse infusions? Have you tried the AGM-1470 in middle-T oncogene-induced transplantable murine hemangiomas? And finally, could you tell us whether AGM-1470 impairs normal wound healing angiogenesis?

P. Donahoe (Boston, MA): Two simple questions. Could you explain why the treated controls get anemia? And also, could that be abrogated with the treatment of erythropoietin?

S. Shochat (Stanford, CA): It is my understanding that you injected the tumor cells and then treated with AGM-1470. Did you allow the tumors to grow and look at treatment after you had a palpable tumor?

L. Hill (Baltimore, MD): For comparison, have you injected or used control groups with steroids or other agents for hemangioma?

M.S. O'Reilly (response): We are currently looking for the presence of mast cells in the hemangioendotheliomas. I suspect that there will be a diminished number of mast cells in the lesions of mice treated with AGM-1470. Recently in our lab, it has been shown that during the normal involution process of some human hemangiomas there is a decrease in the number of mast cells.

Both groups of mice eventually died from hemorrhagic complications. The mice in the AGM-1470 group had prolonged survival but were not cured. Their lesions grew slowly, but all eventually bled.

Brain endothelial cells transfected with the middle-T oncogene (bENDO cells), which have been used in other models of angiomatous disease, formed only small lesions that regressed rapidly in both immunocompetent and immunocompromised mice. As a result, these cells were not used further in our studies.

We have found that AGM-1470 inhibits wound healing if it is started within 5 days of initiation of the wound. After 5 days, the wound healing process does not seem to be affected.

The anemia found in mice implanted with these hemangioendothelioma cells has been described by Hoak et al. These lesions induce a Kasabach-Merritt syndrome that results in hemorrhagic complications.

AGM-1470 therapy was started in mice implanted with hemangioendotheliomas only after tumors had formed and were both visible and palpable.

Some preliminary studies in our lab show that interferon is useful in the treatment of these hemangioendotheliomas. Further, interferon may potentiate AGM-1470 in the treatment of these lesions.